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Quantitative PCR-Enhanced Immunoassay for Measurement of Enteroviral Immunoglobulin M Antibody and Diagnosis of Aseptic Meningitis

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A PCR-enhanced immunoassay (PIA) to detect enterovirus (EV) immunoglobulin M (IgM) for diagnosis of recent EV infection was recently developed. This test was compared with another EV IgM capture technique, the solid-phase reverse immunosorbent test (SPRIST). Fourteen of 43 serum samples from aseptic meningitis patients were positive by PIA, whereas 10 were positive by SPRIST. One of 39 control serum samples was weakly positive by PIA. A single-serum-dilution real-time PCR-based PIA for EV IgM (quantitative PIA [QPIA]) was also developed and evaluated against PIA, SPRIST, an EV IgM radioimmunoassay (RIA), and clinical data. A mixture of 12 EVs was used as the antigen. Results from investigating four groups of serum samples were as follows. (i) The nine PIA-positive serum samples in group 1 were all positive by QPIA. (ii) Group 2 consisted of 59 serum samples from aseptic meningitis patients. Nineteen of 30 serum samples (63%) taken at hospital admission were positive by QPIA. Of these, 17 were positive in EV PCR. (iii) None of the 30 control serum samples in group 3 were positive by QPIA. (iv) For the 24 serum samples in group 4, of which 11 were positive and 13 were negative by RIA, the QPIA results were completely concordant. The sensitivity and specificity of QPIA for diagnosis of EV infection were 70 and 80%, respectively. QPIA provides a rational strategy for the detection of EV IgM, allows the use of viral antigens with minimal purification, and needs no virus-specific reagents apart from those in the PCR. QPIA is a generally applicable method for the detection of viral IgM in IgM capture assays.

Enterovirus (EV) infections are usually asymptomatic or may be manifested by low-grade fever and upper respiratory tract symptoms (13), but they are also the most common etiologic agents of aseptic meningitis (AM), causing as many as 80% of such cases (26). EV infections are also the cause of paralytic poliomyelitis and a major part of myocarditis cases (21). After a viremic phase, specific organ manifestations may ensue in a secondary phase with disease entities such as AM, myocarditis, systemic neonatal infection, and potentially even dilated cardiomyopathy (8) and diabetes mellitus (6). Rapid diagnosis of EV infection allows adequate clinical management (22), and promising developments, such as pleconaril (28), also allow the consideration of antiviral therapy. The importance of diagnosing EV infections is highlighted by recent epidemics with high mortality (5), and surveillance for poliovirus (PV) infection is necessitated by the World Health Organization's aim of eradicating this disease.

Conventional laboratory diagnosis of EV infections is based on detection of the virus in clinical specimens by cell culture, followed by neutralization typing. This method is laborious and time-consuming. Type-specific serological diagnosis of EV-associated disease is also hampered by the existence of at least 72 serotypes, a handful of which may be cocirculating in the population. Cross-reactivity occurs and has been used both in complement fixation tests and in enzyme immunoassays.

Recently, diagnostic tests for EV infections have improved significantly with the advent of broadly amplifying PCR for direct detection of specific EV nucleotide sequences in multiple specimen types (4, 10, 16, 24, 26, 27, 33). Real-time quantitative reverse transcription-PCR (RT-PCR) assays (14), using TaqMan probes (fluorogenic probes based on $5' \rightarrow 3'$ exonuclease activity), have been used to determine the amount of EV RNA in sludge samples (15, 20) and for the detection and quantification of EV in cerebrospinal fluid (CSF) (19). A quantitative single-tube real-time RT-PCR (QPCR) for EV has also been developed recently (18).

EV PCR on CSF may directly detect the cause of AM. However, after clearance of viral RNA, detection of a serological EV antibody response remains a diagnostic alternative. Immunoglobulin M (IgM) antibodies are more useful than neutralizing antibody tests, which mostly measure IgG, because IgM indicates recent infection with a serotype not previously encountered. Specific IgM can often be detected weeks to months after the disappearance of a virus from CSF. The combination of PCR with antibody capture has been used to type EV (32). Immune capture in itself purifies the viral antigen, and the PCR detection step is virus specific. In order to cover a multitude of serotypes with high sensitivity, these principles were utilized in the PCR enhanced immunoassay (PIA), providing a new concept for viral serology, which combines the

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sensitivity and specificity of PCR with an immunoglobulin capture solid-phase immunoassay (1).

The aim of this study was to further develop PIA as a quantitative and sensitive serodiagnostic technique for diagnosing EV infections. Since QPCR greatly simplifies the measurement of virus concentrations, it was logical to extend this methodology to measure viral antigen bound to immobilized patient IgM by taking advantage of both QPCR and PIA in a quantitative PIA (QPIA). Briefly, patient IgM, captured on anti-human IgM-coated microwell plates and thereafter allowed to bind EV RNA released from bound virus by heat denaturation, is used as a template for QPCR.

MATERIALS AND METHODS

Comparison of PIA and SPRIST for detection of EV IgM. PIA was compared with a previously established IgM capture technique, the solid-phase reverse immunosorbent test (SPRIST), for measurement of IgM antibodies to EVs (17) by using serum samples from 43 patients with AM cared for at Uppsala University Hospital (UAS). The sera had been sampled in the convalescent phase during the autumns of 1995 and 1996 and had been stored at $-20°C$. The ages of the patients ranged from 13 to 64 years (mean, 31 years). Thirty-nine blood donor serum samples from UAS served as controls. Virus isolation from CSF had been attempted for 21 patients; 4 of these samples became positive (see below). PIA was performed as described previously (1), except that amplimers were detected by ethidium bromide staining after agarose gel electrophoresis. Prototype strains of coxsackievirus B1 to B5 (CB1 to CB5), coxsackievirus A9 (CA9), and echovirus 6, 11, and 30 (E6, E11, and E30) were obtained from the Swedish National Bacteriological Laboratory (SBL) or from the American Type Culture Collection (ATCC) (ATCC catalog no. VR-28, -29, -30, -36, -41, -184, -185, -186, and -322). The primers used were those of Zoll et al. (33). RT-PCR was done using recombinant *Thermus thermophilus* (r*Tth*) DNA polymerase. The IgM capture step was done in Nunc MaxiSorp microwells coated with anti-human IgM. Serum or blank buffer was added. Bound EV-specific IgM was detected by adding an antigen mixture consisting of 500 50% tissue culture infective doses ($TCID_{50}$) of each of the EVs to each microwell. After thorough washing and lysis of virus, aliquots were transferred to PCR tubes (Microamp; Perkin-Elmer) for RT-PCR.

Amplification products were examined by gel electrophoresis and ethidium bromide staining. Band intensities were scored visually. A 155-bp band of at least the same intensity as the 200-bp band of the DNA 100-bp ladder (1 µl/lane; Amersham-Pharmacia Biotech) was regarded as positive. This scoring system is comparable in sensitivity to the photometric reading of 0.2 absorbance unit used as the cutoff in our previous study (1).

SPRIST was performed as described previously (11, 17). Briefly, IgM from patient serum samples was captured on microhemagglutination plates coated with anti-human IgM. Tissue culture antigen-containing virions (N antigen) and cross-reacting empty capsids (H antigen) from prototype strains of CB3, E3, E5, and E12 were added separately. Bound antigen was indicated by hemadsorption of human red blood cells to the solid phase. If hemadsorption occurred with a serum dilution of 1/100 or more, the result was judged positive.

Patient and control serum samples for QPIA. Four groups of serum samples were investigated. Group 1 contained nine serum samples found to be positive for EV-specific IgM by PIA from a comparison of PIA and SPRIST and were used as positive controls. Group 2 contained 59 serum samples obtained from 1997 to 2001 from patients initially diagnosed with AM at the Infectious Diseases Clinic, Uppsala, Sweden. The ages of the patients ranged from 15 to 77 years (mean, 41 years). These patients had presented with fever, headache, and, in most cases, nuchal rigidity. Their CSF samples had more than 5×10^6 leukocytes/liter. Their diagnoses were retrospectively reevaluated by using patient records and collected laboratory results. Their CSF samples were later tested by QPCR for detection of EV RNA (18). Group 3 contained 30 blood donor serum samples collected in July 2002 which served as controls. Group 4 contained 24 serum samples, 11 from patients with AM and 13 from patients infected with herpes simplex virus, cytomegalovirus, adenovirus, or mumps virus. The EV infection in the patients had been verified by isolation of the virus from CSF, and the samples had been tested previously by an EV IgM radioimmunoassay (RIA) (9). Nearly all serum samples from groups 1, 2, and 4 were drawn for routine diagnosis taken at hospital admission. The control serum samples were analyzed anonymously, as required by ethical rules.

Viral antigen mixture for QPIA. To optimize the antigen mixture and the binding time of the viruses mentioned, nine serum samples (group 1) were tested against the following prototype strains separately: CB1 to CB5, CA9, E6, E11, and E30. The virus mixture later also included Sabin vaccine strains PV1 to PV3, obtained from the SBL. The viral strains were chosen for (i) known cross-reactivity (29) and (ii) frequency and severity of infection. The PV vaccine strains were included to enable the method to be used in current certification of PV eradication.

The infectivities of the virus stocks were measured by determining the $TCID_{50}$ per 0.1 ml in a microtiter plate assay. The titers were calculated according to the Kärber method (12). For propagation and titration, viruses were added to African green monkey kidney cells (Vero cells), obtained from the European Collection of Animal Cell Cultures (ECANN no. 84113001). Virus-infected cell cultures were harvested when complete cytopathic effect was reached. Virus antigen preparations were produced by centrifugation of the culture fluids for 10 min at $440 \times g$. Supernatants were aliquoted and frozen at $-70°C$ until use.

Serial dilution of the EV RNA standard. To calculate the amount of RNA bound per microtiter well for each sample, all QPIA runs included serial dilutions of an EV RNA standard preparation. Serial dilutions of these viruses were made by mixing 100 µl (10,000 $TCID_{50}$) of all viruses (CB1 to CB5, CA9, E6, E11, and E30), to give 140 µl for RNA isolation by minicolumns (QIAGEN).

The resulting 60 µl of EV RNA was diluted 100 times in RNase-free water, aliquoted in 25-µl portions, and then frozen at $-70°C$ until use. The EV RNA standard curve was prepared by 10-fold serial dilutions of 25 µl of RNA from the virus mixture.

IgM capture and binding of viral antigen. The IgM capture step was conducted as follows: microwell plates (Nunc MaxiSorp) were coated with 100 µl of goat anti-human IgM (µ chain specific and affinity purified; Sigma Chemicals, catalog no. 10759), diluted 1:10,000 in phosphate-buffered saline containing 10 mg of sodium azide per liter (PBS), at 37°C on a shaker for 2 h. Wells were blocked overnight with 200 µl of blocking solution (0.1% gelatin in PBS) at 4°C. At this stage, plates could be frozen at $-20°C$ for several months (3). Before use, the wells were washed five times with 0.05% Tween 20 in PBS. One hundred microliters of patient serum diluted 1/200 in a PBS buffer containing 0.1% gelatin and 0.05% Tween 20 was added and incubated on a shaker at room temperature for 1 h. Wells incubated with buffer only were set as negative controls for this step. The serum samples were divided into aliquots and frozen to avoid changes in IgM reactivity that may occur with repeated freeze-thawing. After five washes, 100 µl of an antigen mixture was added and allowed to bind the serum IgM for 1 h at 37°C. The antigen mixture contained 10,000 $TCID_{50}$ of each of the virus strains (CB1 to CB5, CA9, E6, E11, E30, PV1 to PV3). They were diluted in a buffer (PBS containing 0.1% gelatin and 0.05% Tween 20 with 1% fetal calf serum). This corresponded to an approximate dose of 3,000,000 RNA molecules per well. Washing was subsequently performed 15 times with an ELISA Elx50 automated strip washer (Bio-Tek Instruments, Inc. Winooski, Vt.) with an in-line filter (HEPA-VENT; Whatman). The exhaust was further passed through a washing flask containing hypochlorite. The virus was then lysed by the addition of 50 µl of RNase-free water to each well and incubation of the plate for 10 min at 56°C. A 14-µl volume of each sample was transferred to 200 µl of RNase-free PCR tubes (catalog number 3412; Molecular Bioproducts, San Diego, Calif.). QPCR was then conducted as described in the next section. The virus could be stored at $-70°C$ in RNase-free water (Sigma-Aldrich Company) until analysis, without significant loss of RNA (data not shown). However, an uneven loss of RNA, probably due to RNase action, was noted when sample processing was delayed due to a large number of samples. In this case, addition of 10 µl of RNase-free water containing 50 U of RNasin (Promega, Madison, Wis.) after viral lysis could protect the free RNA. Data reported here were means of at least two determinations.

Primers and real-time RT-PCR. Primers and real-time RT-PCR conditions have been described previously (18). Briefly, the TaqMan system consisted of two primers, forward primer NMF1 (5′-GCCCCTGAATGCGGC-3′) and reverse primer NMR1 (5′-AATTGTCACCATAAGCAGC-3′), and a dual-labeled fluorescent TaqMan probe, 5′-6-carboxyfluorescein-CGGAACCGACTACTTTG GGTGTCCGT-dark phosphate-3′ (20). In a volume of 36 µl, the reaction mixture comprised 10 µl of 5×EZ TaqMan buffer; 4 mM manganous diacetate; 400 µM dATP, dCTP, and dGTP; 800 µM dUTP (Applied Biosystems, Stockholm, Sweden); 400 nM primer NMR1; 500 nM primer NMF1; 100 nM probe (Scandinavian Gene Synthesis AB, Köping, Sweden); 10 U of r*Tth* DNA polymerase (Applied Biosystems); and 0.1 U of HK-UNG thermolabile uracil *N*-glycosylase (Epicentre Technologies Corporation, Madison, Wis.). The reaction mixture was added to PCR tubes containing 14 µl of RNA from each sample. Thermal cycling conditions were as follows: 50°C for 2 min, to allow degradation by HK-UNG of any carryover product, followed by 70°C for 10 min to completely

TABLE 1. Comparisons between RNA equivalents bound to IgM by QPIA and PIA titers or RIA titers for serum samples from EV-infected patients[a]

Group and patient no.	Virus isolated	PIA or RIA titer[b]	SPRIST titer[c]	QPIA RNA copies[d]	QPIA value relative to cutoff
Group 1[e]					
13	CB5	*10,000*	100	7,386	15.9
14	ND[f]	*10,000*	Negative	7,873	17.0
15	CB5	*10,000*	Negative	1,606	3.5
18	ND	*10,000*	Negative	891	1.9
26	ND	*10,000*	≥10,000	2,180	4.7
29	ND	*10,000*	100	630	1.4
30	ND	*10,000*	≥10,000	695	1.5
41	ND	*10,000*	Negative	1,905	4.1
43	ND	*10,000*	Negative	3,582	7.7
Group 4[g]					
949	CB4	500		2,832	6.1
1259	CB2	1,000		2,577	5.6
989	CB2	125		846	1.8
1085	CB1	1,000		3,057	6.6
1151	CB2	500		510	1.1
1254	CB1	1,000		1,657	3.6
1280	CB4	125		2,750	5.9
1254	CB1	125		2,517	5.4
1022	CB3	1,000		8,083	17.4
810	CB2, 3	250, 2000		5,511	11.9
1059	CB4	1,000		6,694	14.4

[a] Nine serum samples from group 1 were tested by PIA, and 11 serum samples from group 4 were tested by RIA.
[b] Data for PIA are italicized. PIA titers were 0, 100, and 10,000; RIA steps were 125, 250, 500, 1,000, or 2,000.
[c] SPRIST titers were 0, 100, 1,000, and ≥10,000.
[d] RNA copies of 12 viruses bound per well: CB1 to CB5, CA9, E6, E11, E30, and the Sabin vaccine strains PV1 to PV3.
[e] See the text for details.
[f] ND, not done.
[g] RIA data were obtained by G. Frisk.

inactivate HK-UNG. The UNG inactivation period was followed by 30 min at 60°C for reverse transcription and 5 min at 95°C for initial DNA denaturation and r*Tth* DNA polymerase activation. This was followed by 60 cycles of denaturation at 95°C for 15 s and annealing at 59°C for 1 min.

The hardware and software from the Rotor-Gene 2000 real-time amplification system (Corbett Research, Mortlake, Australia) were used.

Quantification of EV IgM from a single serum dilution. The cycle threshold values of the RNA standard dilutions are plotted against EV RNA equivalents to generate a standard curve. The RNA equivalents roughly correspond to the number of EV RNA copies (18). The standard curve is used to determine the number of EV RNA equivalents bound per well, which is a measure of the amount of EV IgM in the sample.

RESULTS

Comparison between PIA and SPRIST. Fourteen of 43 serum samples (33%) were positive (Table 1). Twenty-one CSF samples were sent for virus isolation, and 4 were positive. In some instances the isolation procedure included verification by PCR. Viral diagnoses were as follows: E9 (patient 5), CB5 (patients 13 and 15), and E30 (patients 19 and 28). One patient was positive for mumps virus IgM. Fifteen of 43 were tested for borreliae; none was positive. SPRIST gave positive results for 10 of 43 samples (23%). Titers of 100 and 10,000 were recorded. PIA titers were 100 to 1,000 in six samples and 10,000 or higher in eight samples (geometric mean log titer, ≥3.8), whereas SPRIST titers were 100 to 1,000 in eight samples and 10,000 in two samples (geometric mean log titer, 2.7). Thus, although the titration steps were not entirely comparable, 5- to 10-fold-higher PIA titers were observed in the concordantly positive samples. Although E30 was part of the antigen mixture

of PIA, both serum samples from patients with E30 infections were negative by PIA. They were, however, weakly positive by SPRIST. The reason may be antigenic drift in the present E30 strain. IgM was detected for 1 of 39 (3%) control samples, at a titer of 1/100, by PIA, for a specificity of 0.97 (38/39). Seven of 43 samples were positive by both PIA and SPRIST, whereas 7 were positive by PIA alone and 3 were positive by SPRIST alone. The two methods thus had a relatively high concordance ($P = 0.0125$ by the chi-square test with Yates' correction).

Optimization of IgM capture. The optimal dilution of anti-human IgM was determined to be 1:10,000 (1). The excellent linearity of the QPCR readout for bound EV RNA (18) should allow the use of only one serum dilution. To determine the optimal patient serum dilution, the nine known EV IgM-positive serum samples were titrated from 1/1 to 1/100,000 (Fig. 1). As noted in the original PIA paper (1), a pronounced prozone effect was present. We therefore had to choose a dilution that was high enough to avoid the prozone while maintaining a high IgM activity. The highest IgM binding was achieved with a serum dilution of 1/100 for all nine serum samples (Fig. 1). By choosing the 1/200 dilution, falsely low values for bound EV RNA due to the prozone could be avoided.

Determination of background reactivity and cutoff level. For determination of the cutoff for the method, the 30 serum samples from blood donors (group 3) were tested (Fig. 2). Based on these results, a cutoff level of 2 standard deviations (SD) above the average of the QPIA values obtained from blood donors and wells without serum was calculated. These
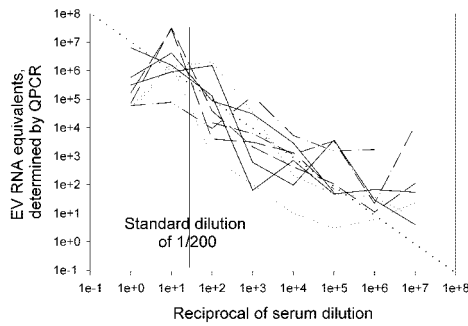
FIG. 1. The highest IgM binding was achieved with a serum dilution of 1/100. Nine known EV IgM-positive serum samples were diluted up to 10 million times. Dotted line, theoretical curve for a signal proportional to dilution. The chosen standard dilution of 1/200 is indicated.

were used together to calculate the background mean, since they gave approximately the same values. In several runs, the SD was 70% of the background mean. The cutoff was therefore subsequently calculated as 2.4 times the mean between the background binding of the EV mixture to one well mock incubated with serum dilution buffer and the binding of another well incubated with serum from a blood donor.

To test the possible influence of PV antibodies on EV-specific IgM, we tested the serum samples of the 30 blood donors by QPIA with an antigen mixture containing only the three PV vaccine strains. None were positive. The possibility that recent PV vaccination gives false-positive results remains.
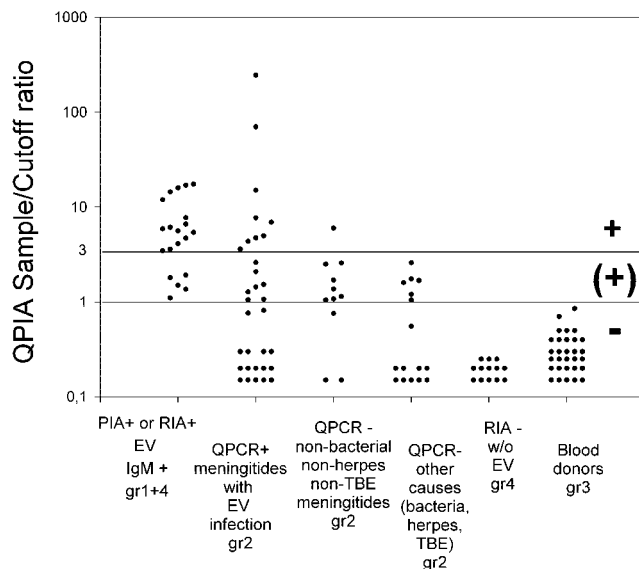

FIG. 2. Distribution of QPIA binding values in four categories of serum samples. Serum samples from 20 patients previously found to be EV IgM positive (groups [gr] 1 and 4), 59 meningitis patients (group 2), and 30 blood donors (group 3) were tested. The 59 group-2 samples were divided into samples from 32 meningitides with known EV infection (QPCR positive in CSF), 11 meningitides for which the cause was unknown (QPCR negative in CSF), and 16 meningitides with known or possible bacterial, herpes simplex, or TBE infection. Results of 13 RIA-negative samples (group 4) are also shown. Values between 0 and 0.5 are plotted only to show the number of observations.

As shown in Fig. 2, serum samples from some non-EV meningitis cases were positive by QPIA. However, they all gave an EV binding value of <3 times the cutoff. A second cutoff level was therefore defined. EV binding between 1 and 3 times the cutoff was termed "low EV IgM levels," whereas binding of >3 times the cutoff was termed "high EV IgM levels."

Comparison of QPIA with other EV IgM techniques. For the serum samples of group 1, the QPIA values were compared with values from PIA and SPRIST. Nine serum samples were positive by PIA (patients 13, 14, 15, 18, 26, 29, 30, 41, and 43), and 4 were positive by SPRIST (patients 13, 26, 29, and 30). QPIA gave a positive reaction with all nine IgM-positive serum samples (Table 1).

The sensitivity of QPIA was tested using serum samples from patients with AM from which EV had been isolated (group 4). The QPIA values were compared with values from RIA (9) (Table 1). RIA detected EV-specific IgM in 11 serum samples at different titers, and QPIA gave a positive reaction for all. Nine of the 11 were strongly positive. QPIA gave a negative result for 13 serum samples from persons unlikely to be EV infected. All of them were negative by RIA. The association between QPIA and RIA was highly statistically significant ($P < 0.0001$ by the chi-square test with Yates' correction). None of the blood donor serum samples (group 3) had EV IgM by QPIA, indicating a high specificity.

To study the contributions of the individual EV strains to the amount of EV RNA bound from the mixture, the nine group-1 serum samples were run separately with each of nine EV ATCC reference strains. Although the EVs that gave rise to IgM were not known, it is reasonable to assume that most patients in the same season were infected with the same viral strain. The serum samples were obtained from AM cases in Uppsala during the autumns of 1995 and 1996, when E9, CB5, and E30 had been isolated from AM cases in this city (Fig. 3). Figure 3 demonstrates a high degree of cross-reactivity between CB2, CB4, and E30. The high level of binding of E30 in the serum samples from the nine patients (patients 13, 14, 15, 18, 26, 29, 30, 41, and 43) by QPIA contrasted with the absence of binding by PIA and weak binding by SPRIST to E30 in samples from two patients from whom E30 was isolated (patients 19 and 28). These samples were not tested by QPIA. This inadvertence is discussed in the Discussion.

Comparison of QPIA and QPCR against clinical and microbiological diagnosis. In group 2, containing 62 CSF samples, 45 patients were finally diagnosed with AM not due to bacteria, herpes simplex, or tick-borne encephalitis (TBE). Seventeen patients were diagnosed with non-EV infection. Of these, nine patients were considered to have "bacterial or possibly bacterial" meningitides: six whose CSF samples showed growth of *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Neisseria meningitidis* and three who concomitantly had other diseases or conditions (epilepsy, cystitis, or central nervous system surgery). The causes of the latter meningitides were unknown, but they may have been of bacterial origin. In seven cases, CSF was positive for herpes simplex virus type 2 by PCR; several of these patients had recurrent meningitis (Mollaret's disease). In one case TBE was diagnosed by serology. These diagnoses were based on routine diagnostic tests at the local or another virological laboratory. As reported previously,
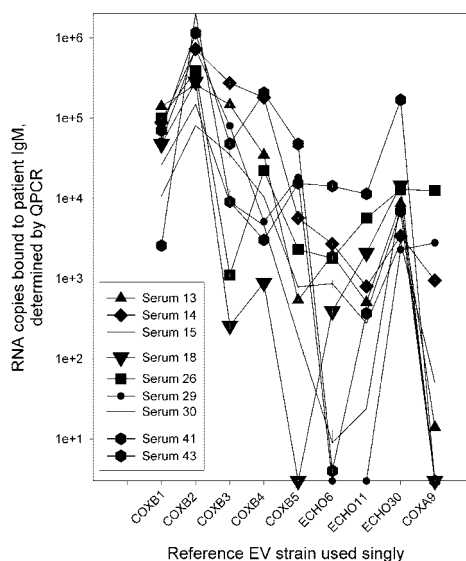
FIG. 3. Contributions of individual EVs to the amount of EV RNA bound from the viral antigen mixture. The nine serum samples were run with nine EVs, but not with PV1, -2, or -3. CB2, CB1, and E30 dominate the binding. The serum samples were obtained from AM patients during the autumns of 1995 and 1996, when E9, CB5, and E30 had been isolated from AM patients in Uppsala.

34 of the 45 (76%) CSF specimens were positive by QPCR (18).

Serum samples were available for 59 of the 62 patients, and for 43 of the 45 patients finally diagnosed with AM not due to bacteria, herpes simplex, or TBE. Sixteen of the remaining 17 patients were diagnosed with non-EV infections. QPIA was positive for 24 of the 43 (56%) cases. However, samples from four cases finally diagnosed as bacterial or probable bacterial meningitis were also (weakly) positive by QPIA. One sample weakly positive by QPIA was from a case of herpes simplex type 2 meningitis, and one was from a case of TBE. Nineteen out of 30 (63%) serum samples obtained on the day of hospital admission were QPIA positive. Of these, 17 were positive for EV by PCR. Sixteen of 43 samples were positive by both QPCR and QPIA. These included samples from two patients from whom EV (E6 and E30) had been isolated. Sixteen of 43 samples were positive by QPCR alone, and 8 of 43 were positive by QPIA alone.

Altogether, 18 QPCR-positive patients tested negative by QPIA. These included three patients from whom EV (CB5, E9, or E33) was isolated. The combination of QPCR and QPIA detected 40 of 43 (93%) AM cases when both serum and CSF were available.

A specificity of 63% and a sensitivity of 56% were obtained for the QPIA for detection of EV-specific IgM when the results were compared against clinical data indicating an EV infection. For any particular positive result, the probability that it was a true positive was 80% (probability of a false positive, 20%); the probability of a negative result being a true negative was 34% (probability of a false negative, 66%) (calculated by using the online clinical calculator at http://faculty.vassar.edu/lowry/clin1.html). However, a larger number of samples would

have been desirable for a more definite statement on these probabilities.

Sensitivity, specificity, and reproducibility of QPIA. The clinical utility of a diagnostic test finally depends on its ability to detect or predict disease. For all 122 samples (44 QPIA positive with EV disease, 19 QPIA negative with EV disease, 6 QPIA positive without EV disease, and 53 QPIA negative without EV disease), the sensitivity and specificity of QPIA versus both laboratory and clinical data became 70 and 90%, respectively. The predictive value of a positive QPIA result was 88%, and that of a negative value was 74% (calculated by using the online clinical calculator at http://www.intmed.mcw.edu/clincalc/bayes.html).

Reproducibility was established by testing 11 positive serum samples from group 4 and repeating the test on a different day. The means for the two runs were 3,057 and 3,290 bound RNA equivalents, respectively. The SD between results of individual samples of the two runs was 5.2, giving a coefficient of variation of 0.16%. This is unexpectedly small, because quantitative PCR normally has a greater variance. However, it indicates that with experience, QPIA is a precise and reproducible technique.

DISCUSSION

Promising developments in antipicornavirus drugs (7, 23, 30) highlight the need to diagnose EV infections early in the course of the disease. PIA, which combines the sensitivity and specificity of PCR with an immunoglobulin capture solid-phase immunoassay, is at least as sensitive as SPRIST yet has a low background reactivity in negative-control serum samples. SPRIST is a less expensive technique than PIA, but PIA is more versatile, because it does not require hemadsorption and requires only small amounts of virus, which can be taken from infected-tissue culture supernatants. The enzyme immunoassay-like quantification step in the original PIA procedure is relatively laborious. We did not use it for the present investigation. Instead, we scored the intensities of bands in agarose gel electrophoresis at serum dilutions of 1/100 and 1/10,000. The subsequent evolution of PIA into QPIA gave a more rational technique due to the use of real-time PCR (14). We judge that PIA and QPIA are sufficiently functionally similar to conclude that the sensitivity and specificity of the PIA techniques are similar to those of SPRIST.

The QPIA combines solid-phase serological techniques with QPCR. The QPCR uses the dual ability of the enzyme r*Tth* to act as a reverse transcriptase as well as a DNA-dependent DNA polymerase. Moreover, it uses the ability of HK-UNG to minimize the risk of amplimer carryover contamination in QPCR.

The success of an immunoassay depends on choosing a good antigen. The PIA for detection of EV IgM was developed to detect antibodies against a broad range of native EV antigens (1). In QPIA the same antigens were used, but the reference Sabin vaccine strains PV1 to PV3 were added. Although serum samples from actual poliomyelitis cases were not tested, the inclusion of PV antigens makes it likely that recent PV infections will also be detected by the QPIA. This assay should therefore be considered an aid to current efforts to eradicate poliomyelitis. The addition of PV did not increase background (i.e., blood donor or non-EV meningitis) reactivity appreciably

(data not shown). Remaining PV vaccine-induced IgM reactivity is not a likely cause of false-positive QPIA EV IgM values for Swedish adults. We did not investigate if recent PV vaccination might give false-positive results. Each EV strain in the antigen mixture was titrated to achieve similar RNA contents, meaning similar numbers of complete virus particles for all strains. The antigen mixture can easily be modified. A single infected-tissue culture tube can provide enough antigen for several years. However, viral antigen variation is a well-known problem for many viral serological methods. The ATCC EV strains, which we demonstrate to work, were obtained at geographically different sites, several decades prior to the EV infections which we investigated. Thus, it is probable that an EV mixture for QPIA can be kept for a reasonably long period.

The use of a single serum dilution is rational. We demonstrated for nine serum samples that the EV binding values obtained at a single serum dilution of 1/200 are roughly representative of the binding at higher dilutions. Although we obtained indications that the number of RNA equivalents bound in QPIA at this serum dilution was representative of binding at lower antibody concentrations, the linearity between titer and virus binding at 1/200 was not rigorously tested. Such testing should be done in a larger follow-up study.

The two PIA-negative E30-infected patients merit a comment. IgM will arise only in response to EV epitopes not previously encountered by the patient. If the patient was infected by an E30-like virus earlier, only a few neoepitopes may give rise to IgM. Those particular epitopes may not be present in the EV antigen mixture of PIA. The two E30-infected persons were adults. It is reasonable to expect that the EV IgM response gradually narrows as the patient encounters new EV strains. A different E30 strain was used in QPIA. In the QPIA evaluation, E30 was one of the most frequently antigenic strains. Thus, the influence of strain differences in the antigen mixture should be a subject of further investigation.

Judging from the results with the nine PIA-positive serum samples, QPIA seems to have approximately the same sensitivity as the previous PIA method and SPRIST (1). The concordance with RIA was high for 24 serum samples. The RIA method has been evaluated thoroughly in clinical practice for more than a decade (9). RIA uses broadly antigenically reactive procapsids lacking nucleic acid and cannot be used in QPIA. We tried to compensate for the narrower antigenicity of virions by including a greater variety of strains in the QPIA. Despite this difference, a high concordance between QPIA and RIA was observed. More-thorough evaluations of QPIA versus other EV IgM methods have to be performed in the future.

The humoral immune response is a secondary phenomenon compared to direct viral detection. Therefore, serological techniques can never be 100% specific and 100% sensitive. In our case, QPIA was 70% sensitive and 90% specific for EV disease, and a positive test had a positive predictive value of 88%. On the positive side, none of the 30 blood donor serum samples in group 3 had EV IgM. However, EV IgM determination has limitations, and false-negative as well as false-positive results are expected to occur (2). Not only sampling time but also the previous EV infection record of the individual and viral antigenic variation will influence the result. On the other hand, serum samples are easier to obtain than other samples, and a likely diagnosis may be obtained retrospectively. It was therefore not surprising that QPIA gave positive results for only half of the QPCR-positive patients in group 2. Conceivably, this was due either to a too-early sampling time, an inability to detect IgM against all EV strains, or the fact that some EV-infected patients did not produce cross-reacting EV IgM (2).

It is well known that, compared to that in the first days of EV disease, the frequency of EV IgM increases in the second and third week after the onset of disease (17). In group 1 we had only one serum sample from each patient, except in one case where a second serum specimen had been taken 3 months after the onset of disease. Although both serum samples tested positive, the patterns of binding of individual EVs differed (data not shown) (even if splitting of the antigen mixture into its components is possible in QPIA, we did not pursue this possibility here). In group 1, in most cases, the serum sample was taken 0 to 2 days after the onset of symptoms. On the other hand, QPIA gave weakly positive results for a few AM cases in group 2 with probable EV etiology, where QPCR did not detect EV RNA. QPIA for detection of EV IgM gave weakly positive results for a few patients suffering from non-EV meningitis and negative results for a few known EV meningitis cases. In other EV IgM assays, EV IgM is known to persist after a recent EV infection (11, 21). EV typically gives clinical or subclinical ratios of 1/100 (31). Thus, a potential problem with serum samples taken during the season of high endemicity, in summer and autumn, would be an increased level of EV IgM in the population. The general EV IgM level and EV strain spectrum will also differ between years. This divergence may have contributed to the weakly positive QPIA results in group 2, some of which are probably false positives. High EV IgM values, more than three times higher than the cutoff, occurred mainly in EV QPCR-positive patients. By use of high EV IgM levels as a criterion for EV infection, the combination of high EV IgM levels and QPCR allowed an EV diagnosis in almost all cases of nonherpes, non-TBE AM. The main strength of QPIA probably is that it allows a likely retrospective diagnosis of EV infection in strongly IgM positive meningitis cases when PCR in CSF gives a negative result. A more presumptive diagnosis is provided in weakly positive cases.

Among the 12 EV strains included in QPIA are the CB, E11, and E30 strains, which have remained dominant, both epidemiologically and pathogenically, in many societies for many decades (25). However, the QPIA-negative, EV-positive cases may indicate that a further revision of the EV antigen mixture could increase the QPIA positivity rate in EV infections. Such optimization is, however, quite laborious. We judge that the chosen mixture is sensitive enough for most EV infections.

In contrast to other methods for demonstration of the presence of viral IgM, QPIA obviates additional detection steps using antiviral antibodies, chemically coupled virus antigen conjugates, or isotope-marked virus antigens. For a laboratory with an established EV QPCR technique, QPIA is thus a more flexible method, less dependent on complex commercial reagents than enzyme immunoassay-based methods. Once a QPIA method has been established, it is easy to adapt to measurement of IgM against other viruses. Any virus for which there is a QPCR and which can be cultivated can be used for QPIA. However, detergent must be excluded from the wash buffer if IgM to an enveloped virus is to be measured.

In conclusion, judging from the limited number of samples

tested by other EV IgM techniques, QPIA was sensitive and specific. The results indicate that the QPIA technique can be used for the detection of EV infections. EV RNA detection and EV IgM detection are complementary and, used together, increase the chance of establishing a diagnosis of EV infection. Further advantages of QPIA are that EV QPCR and QPIA can be run together in the same real-time PCR machine and that QPIA allows quantitative assessment of the amount of EV IgM without titration. The quantification allows a comparison of IgM levels at multiple sampling times, which can enhance diagnostic accuracy.

Although real-time PCR uses some costly reagents at present, it is likely to become much less expensive in the future. It is becoming a major technique in microbiological laboratories. QPIAs for IgM detection can be set up relatively easily for viruses for which there is a real-time PCR assay. We therefore think that the QPIA method is of great interest for many clinical and research laboratories. If the quality of the real-time PCR method is ensured, and QPIA is further standardized, the accurate measurement of bound virus particles may make the corresponding QPIA suitable as a reference viral IgM method.

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